



## Slowly-Evolving Protein Loci and Higher-Level Snake Phylogeny: A Reanalysis

Larry Buckley; Maureen Kearney; Kevin de Queiroz

*Herpetologica*, Vol. 56, No. 3. (Sep., 2000), pp. 324-332.

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rona Santiago: Río Piuntza, 1830 m, KU 147071–72, 147074, 147076–77, 147079.

*Atelopus carrikeri*.—COLOMBIA: *Departamento Magdalena*: Sierra Nevada de Santa Marta, Cuchilla Cebolleta, ICN 32429 (C&S); *Departamento Guajira*: Santa Marta mountains, Páramo de Macotama (2430–4570 m elevation), UMMZ 48273 (paratype); *Departamento del César*: Río Guatapurí, Sierra Nevada de Santa Marta, USNM 123561–62.

*Atelopus exiguus*.—ECUADOR: *Provincia Azuay*: Zurucuchu, W of Cuenca, 3250 m, SMF 4046 (lectotype), 4047–51, 3170–71, 3916 (paralectotypes); Laguna de Zurucuchu (= Laguna Llaviuco), 3200 m, KU 120381 (cleared and stained), 120385 (cleared and stained), 120387, QCAZ 3744; 10 km W Cuenca, QCAZ 4957 (cleared and stained); Río Quinuas, 15 km W Cuenca, 3150 m, QCAZ 3668 (12 tadpoles), QCAZ 3669 (1 tadpole); Parque Nacional Cajas, 15–20 km W Cuenca, 3200 m, MHNG 2499.72–75,

2499.76 (cleared and stained), 2499.77–79; Bosque de Mazán, QCAZ 1666; Lagunas de Cajas, 3850 m, MHNG 2258.41–42; Girón, Quimsacocha (páramo sur del Cajas), 3700 m, QCAZ 8835.

*Atelopus ignescens*.—ECUADOR: *Provincia Imbabura*: Lagunas de Mojanda, QCAZ 1852–56; *Provincia Napo*: Oyacachi, QCAZ 275–76; *Provincia Pichincha*: Páramo de Guamaní, La Virgen, 3800–4200 m, MHNG 2410.1–6, 2409.95–100, 2273.48, 2273.61, 2273.81–82, 2273.84–97; QCAZ 266 (cleared and stained); *Provincia Cotopaxi*: Laguna de Limpiopungo, 4000 m, QCAZ 254, 385–86, 8797; Zumbagua, MHNG 2384.97–100, 2385.1–10; *Provincias Cotopaxi-Napo*: ca. 20 Km (air-line) southeast of Latacunga, on road from San Miguel de Salcedo to Lagunas de Antojos and towards the east, 01° 01' S and 78° 25' W, approximately between 3200–3800 m, QCAZ 702 (neotype), 703–16, 1857–58; *Provincia Chimborazo*: 20 km N Riobamba, QCAZ 9; *Provincia Bolívar*: ca. Chimborazo (Ambato-Guaranda road), QCAZ 641.

*Herpetologica*, 56(3), 2000, 324–332  
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## SLOWLY-EVOLVING PROTEIN LOCI AND HIGHER-LEVEL SNAKE PHYLOGENY: A REANALYSIS

LARRY BUCKLEY<sup>1,3</sup>, MAUREEN KEARNEY<sup>2</sup>, AND KEVIN DE QUEIROZ<sup>3</sup>

<sup>1</sup>*Biology Department, Rochester Institute of Technology, Rochester, NY 14623, USA*

<sup>2</sup>*Department of Biological Sciences, George Washington University, Washington, DC 20052, USA*

<sup>3</sup>*Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC 20560, USA*

**ABSTRACT:** We reanalyzed data from a recently published study of higher-level snake relationships based on four slowly-evolving protein loci. The original study used phenetic clustering of genetic similarities and presented a single, highly resolved tree. Our reanalyses of these data reveal that the single published phenogram is only one of at least 10,000 equivalent UPGMA phenograms, the consensus of which is largely unresolved. Additive distance analysis and character-based parsimony analysis of the data also yield little resolution, indicating that these data are highly ambiguous regarding higher-level snake phylogeny. The high degree of resolution in the published phenogram is an analytical artifact resulting from the failure to consider alternative trees implied by tied distance values, which are numerous in the distance matrix derived from this particular data set. Although the published phenogram exhibits general agreement with traditional hypotheses about snake relationships, the same appears to be true for the thousands of equivalent phenograms, discrepancies among which sum to a substantial loss of resolution. Although the four loci sampled are evolving slowly relative to other commonly surveyed protein loci, they are nevertheless evolving too rapidly to be informative about the higher level phylogeny of snakes.

**Key words:** Allozymes; Genetic distances; Minimum evolution; Parsimony; Phenetic clustering; Phylogeny; Serpentes

IN a recent study of the higher-level relationships among snakes, Dowling et al. (1996) analyzed genetic similarities based on four protein-coding loci using average linkage phenetic clustering, specifically,

the unweighted pair group method using arithmetic averages (UPGMA). Their result was a single, highly resolved tree (Fig. 1) that delineated 103 groups and exhibited general agreement with traditional

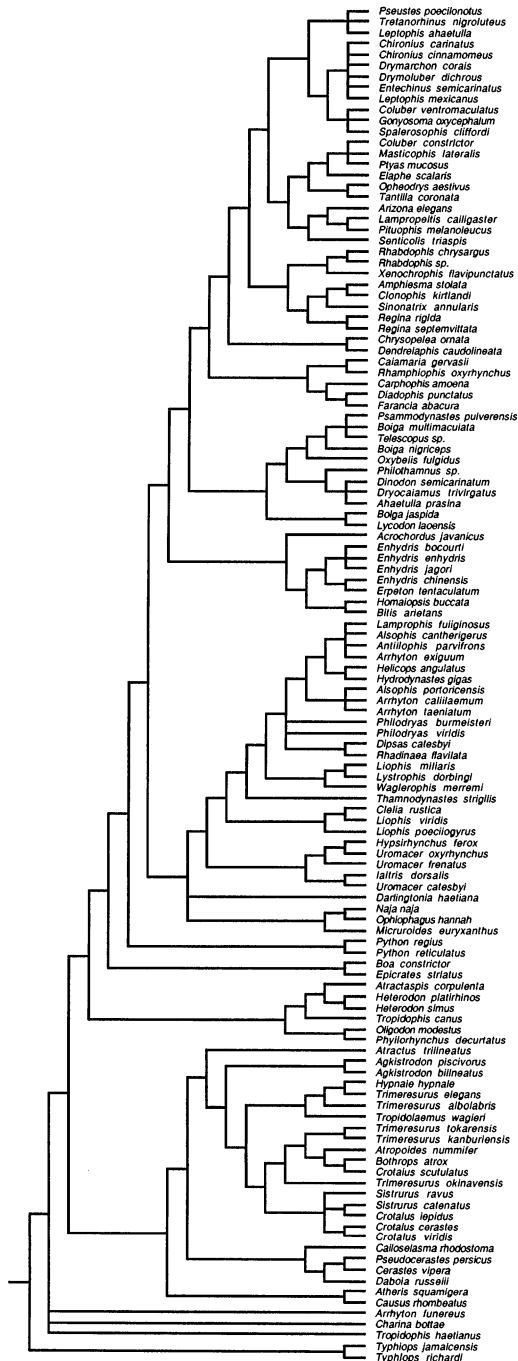


FIG. 1.—Published phenogram of Dowling et al. (1996) with identical taxa removed and with *Uromacer catesbyi* (b) replaced by *Uromacer oxyrhynchus*, in agreement with Table IV of Dowling et al. (1996). This dendrogram represents only the topology, and not the branch lengths, of the published tree. According to the TAXAN output (supplied by C. Hass),

taxonomic groupings. The authors argued that the slowly evolving nature of the loci and their ability to obtain traditionally recognized groups supported the systematic utility of such data. Here we show that the highly resolved tree presented by Dowling et al. (1996) is an analytical artifact. We reanalyze their data and show, using several different analytical approaches, that those data are highly ambiguous, supporting thousands of trees equally well. Our results call attention to the artifactual resolution that can result from failing to consider ties in a distance matrix as well as the ambiguity of data consisting of relatively few loci each with numerous alleles. Our results also call into question the slowly evolving nature of the four protein loci sampled relative to the time interval occupied by the diversification of snakes.

METHODS AND RESULTS

The Data

Dowling et al. (1996) collected data on four protein-coding loci (*Acp-2*, *Ldh-2*, *Mdh-1*, *Pgm*), each of which exhibited numerous alleles (42, 43, 29, and 25, respectively). They sampled 216 snake species, each represented by a single specimen (two alleles). Degrees of resemblance among taxa were estimated using Nei's (1972) coefficient of genetic identity (*I*). Because the similarity matrix was not published, and because some of the programs we used required distances, we first calculated Nei distance values from the allele frequency data (Dowling et al., 1996: their Tables I, II, III, IV, and V), using the GENDIST program in PHYLIP version 3.5c (Felsenstein, 1993). We then con-

three of the polytomies in this tree are actually resolved: (1) *Darlingtonia haetiana* clusters with the elapids (*Naja naja* through *Micruroides euryxanthus*) before clustering with the other xenodontines (*Lampropeltis fuliginosus* through *Uromacer catesbyi*); (2) *Sistrurus ravus* clusters with *S. catenatus* and *Crotalus lepidus* before clustering with *C. cerastes* and *C. viridis*; and (3) the group composed of all taxa from *Pseustes poecilonotus* through *Causus rhombeatus* clusters first with *Arrhyton funereus*, second with *Tropidophis haetianus*, and third with *Charina bottae*.

TABLE 1.—Summary of distance ( $D = 1 - I$ ) values and their frequencies of occurrence for the snake allozyme data of Dowling et al. (1996). Values in bold correspond with the five fractions of alleles shared by specimens that are homozygous at all four loci (i.e., 0/4, 1/4, 2/4, 3/4, 4/4).

Distance value	Number of occurrences in matrix (all taxa)	Fraction of total occurrences (all taxa)	Number of occurrences in matrix (identical taxa eliminated)	Fraction of total occurrences (identical taxa eliminated)
<b>1.0000</b>	<b>7716</b>	<b>0.518618</b>	<b>4357</b>	<b>0.571335</b>
<b>0.7500</b>	<b>4450</b>	<b>0.299099</b>	<b>2019</b>	<b>0.264752</b>
<b>0.5000</b>	<b>1199</b>	<b>0.080589</b>	<b>470</b>	<b>0.061631</b>
0.8664	539	0.036228	362	0.047469
<b>0.2500</b>	<b>403</b>	<b>0.027087</b>	<b>105</b>	<b>0.013769</b>
0.5991	165	0.011090	106	0.013900
0.7327	140	0.009410	102	0.013375
<b>0.0000</b>	<b>131</b>	<b>0.008805</b>	<b>0</b>	<b>0.000000</b>
0.3318	35	0.002352	22	0.002885
0.0646	22	0.001479	9	0.001180
0.9286	14	0.000941	14	0.001836
0.4655	9	0.000605	9	0.001180
0.8557	8	0.000538	8	0.001049
0.8571	7	0.000470	7	0.000918
0.2783	6	0.000403	4	0.000525
0.7113	5	0.000336	5	0.000656
0.5670	5	0.000336	4	0.000525
0.3571	3	0.000202	3	0.000393
0.1339	3	0.000202	2	0.000262
0.4286	3	0.000202	3	0.000393
0.7143	2	0.000134	2	0.000262
0.6429	2	0.000134	2	0.000262
0.3828	2	0.000134	2	0.000262
0.1982	2	0.000134	2	0.000262
0.2857	1	0.000067	1	0.000131
0.6914	1	0.000067	1	0.000131
0.4226	1	0.000067	1	0.000131
0.2285	1	0.000067	1	0.000131
0.0742	1	0.000067	1	0.000131
0.5714	1	0.000067	1	0.000131
0.0714	1	0.000067	1	0.000131
Totals	14,878	1.000000	7626	1.000000

verted the Nei distances to Nei identities and the Nei identities to their complement distances using the equation  $D = 1 - I$ , which yielded distances from 0 (all alleles identical) to 1 (no alleles shared) for all pairwise comparisons. We used  $D = 1 - I$  rather than the standard Nei's  $D$  ( $= -\ln I$ ) because distance clustering on  $D = 1 - I$  values (but not necessarily on Nei's  $D$  values) should give the same results as similarity clustering on Nei's  $I$  values. Before calculating the identities and distances, we corrected two typographical errors in the data tables published by Dowling et al. (1996): the allele for *Acp-2* in *Tropidophis canus* was corrected from 03 to 42 and the allele for *Pgm* in *Sistrurus caten-*

*atus* was corrected from 15 to 22 (original data provided by C. Hass and R. Highton).

Table 1 summarizes the distance values and their frequencies of occurrence. Of the 14,878 values (pairwise comparisons) in the matrix, 14,871 (99.9%) are tied with at least one other value. The great majority of these ties (93.4% of the total values) represent values corresponding with the five possible fractions of alleles shared by specimens that are homozygous for all loci; that is, 4/4 (0.9%), 3/4 (2.7%), 2/4 (8.1%), 1/4 (29.9%), and 0/4 (51.9%). The remaining values (6.6%) are accounted for by comparisons involving heterozygotes. Because identical taxa ( $D = 1, I = 0$ ) provide no additional information about the struc-

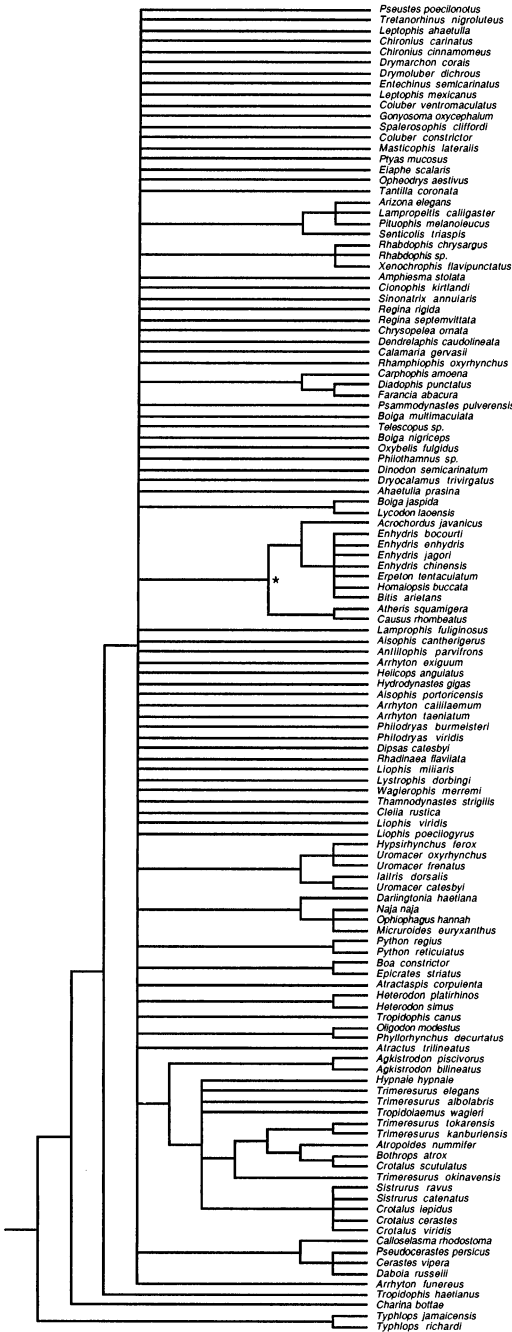


FIG. 2.—Strict consensus tree for equivalent UPGMA trees resulting from alternative clustering pathways caused by ties in the distance matrix. The consensus tree is based on 9999 equivalent UPGMA trees found by systematic tie-breaking using NTSYSpc. An asterisk indicates an additional node that is collapsed when the published tree of Dowling et al. (1996) is included in the set of alternative trees,

ture of the tree, they were excluded from the remainder of our analyses. The reduced matrix has 124 taxa, 7626 pairwise comparisons, and still contains 7619 values (99.9%) that are tied with at least one other value (Table 1). Once again, the great majority of the total values (91.1%) correspond with the five possible fractions of alleles shared by homozygotes.

Phenetic Clustering

Identical distance values (i.e., ties) imply the existence of equivalent phenograms (trees generated by phenetic clustering), which are roughly analogous to equal minimum-length trees that are commonly found in parsimony analyses (Hart, 1983; de Queiroz and Good, 1997). Because there are numerous ties in the snake distance matrix, we performed a UPGMA analysis with NTSYSpc 2.01d (Rohlf, 1996) using the FIND option to search for tied (equivalent) trees with the tie tolerance (TOL) set to the default value of 10<sup>-10</sup>. The search ended at 9999 trees, the program maximum, which was increased from 999 at our request by F. J. Rohlf. The strict consensus of these tied trees (Fig. 2) has only 34 nodes, in contrast with the 103 nodes in the published tree (Fig. 1). Given that the program reached its limit for tied trees, the existence of additional equivalent trees and an even less resolved consensus tree is likely.

Of the 34 nodes in the consensus tree (Fig. 2), most (85%) unite relatively small numbers of taxa (2–10) and reflect relatively shallow divergences; relationships corresponding with early cladogenetic events are largely unresolved. Based on the topology of the consensus tree, none of the 9999 trees corresponds exactly with the published tree of Dowling et al. (1996); specifically, our analysis failed to reproduce the placement of the group composed of *Causus* and *Atheris* in their tree (other seeming discrepancies between

← which is the same additional node that is collapsed in the consensus tree for the 1000 equivalent UPGMA trees found by random tie-breaking using PAUP\*.

our consensus tree and their published tree—involving the positions of *Darlingtonia*, *Tropidophis haetianus*, and *Charina*—result from false polytomies in their tree; see Fig. 1 legend). Assuming that their tree represents an additional equivalent UPGMA tree, then including it in the consensus would result in the loss of an additional node (marked with an asterisk in Fig. 2). Dowling et al. (1996) used a different order of taxa in their datafile (supplied by R. Highton) than that published in their tables; we used the order in their tables. This difference combined with the fact that our analysis did not find all of the equivalent UPGMA trees presumably accounts for the minor discrepancy between the consensus of our 9999 UPGMA trees and their published tree.

Because NTSYS generates equivalent phenograms by systematically exploring the alternative clustering pathways resulting from ties, we also used the random tie-breaking procedure in PAUP\* version 4.0b2 to examine alternative phenograms. We performed 1000 UPGMA analyses, each with a different random number as a tie-breaking seed, producing 1000 equivalent UPGMA trees. The strict consensus of these trees had 33 resolved nodes, one node fewer than in the consensus of 10 times as many trees resulting from systematic tie-breaking using NTSYS (Fig. 2) and differing from that tree in the absence of the node uniting *Causus* plus *Atheris* with *Acrochordus* and the homalopsines. Because the number of nodes in the consensus tree for the analysis using random tie-breaking is less than the number of nodes in the consensus tree for the analysis using systematic tie-breaking, there must be more than 9999 equivalent UPGMA trees. In addition, absence of the node uniting *Causus* and *Atheris* with *Acrochordus* and the homalopsines removes the only discrepancy between our UPGMA consensus tree and the tree obtained by Dowling et al. (1996).

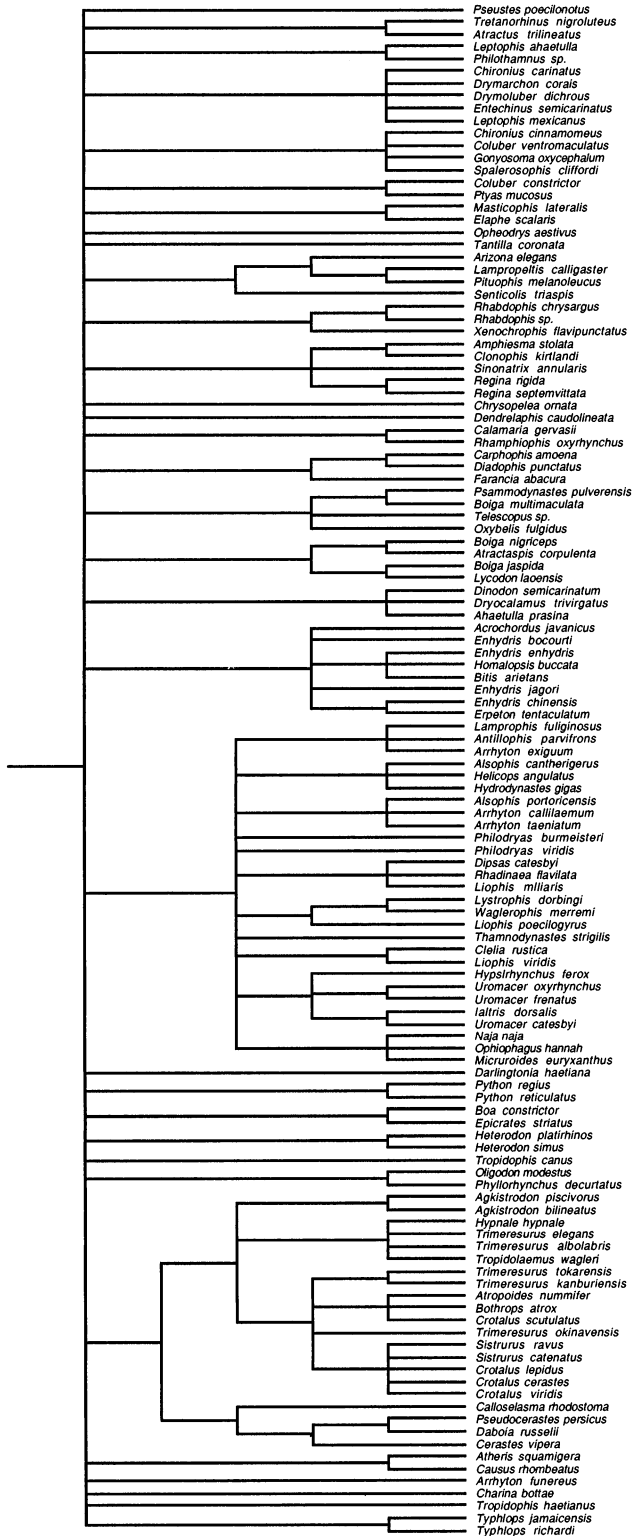
### Additive Distance Analysis

When used to estimate phylogeny, phenetic clustering methods such as UPGMA carry an implicit assumption that rates of evolution among lineages are roughly constant (reviewed by de Queiroz and Good, 1997). Moreover, those methods do not employ an optimality criterion, making evaluation of alternative topologies impossible. Therefore, we also analyzed the data using two alternative approaches: additive distances and parsimony (reviewed by Swofford et al., 1996). For the additive distance analysis, we used the minimum evolution criterion in PAUP\* test version 4.0b2. A heuristic search was performed on the distance matrix, obtaining the starting tree by neighbor-joining and searching for shorter trees using tree-bisection-reconnection (TBR) branch swapping. We saved all trees shorter than the initial neighbor-joining tree (length = 28.54687), which was itself shorter than the published UPGMA tree (length = 28.87127). The search was terminated at 233,363 trees, but even more such trees presumably exist, because the search was not completed. The strict consensus of these 233,363 trees (not shown) has only seven resolved nodes, each of which unites only two terminal taxa. Thus, under the minimum evolution criterion, there are hundreds of thousands of trees shorter than both the neighbor joining tree and the published tree, one or more of which contradict all but seven of the nodes in the published tree.

A second minimum evolution analysis was performed, once again obtaining the starting tree by neighbor-joining and using TBR branch swapping, but this time keeping only the best trees. This analysis yielded six optimal trees (length = 28.17102), the strict consensus of which (not shown) is highly resolved (120 nodes). Much of that resolution, however, is of questionable significance. The smallest distance in the data matrix (0.0646; Table 1) corresponds

→

FIG. 3.—Strict consensus of six optimal additive distance trees (minimum evolution criterion) with branches of length less than 0.0646 collapsed (see text for explanation).



with the difference between taxa that possess the same single allele at each locus, except for the presence of a second allele in one of the taxa at one of the four loci (because the taxa are represented by single specimens, this difference corresponds with the difference between an individual that is homozygous for all four loci and one that is homozygous for the same allele for three of those loci but heterozygous for the fourth, with one of the two alleles of the heterozygote shared with the allele in the homozygote). If this distance (0.0646) is taken as the smallest distance that has a meaningful interpretation in terms of allelic change, and all branches shorter than it are collapsed, the resulting tree (Fig. 3) is considerably less resolved (55 nodes) and exhibits little deep structure.

#### *Parsimony Analysis*

We performed a parsimony analysis of the data using PAUP<sup>®</sup> 4.0b2, treating the loci as characters and alleles as unordered states (e.g., Buth, 1984; Mickevich and Mitter, 1983). Multistate taxa (heterozygotes) were treated as polymorphic. In contrast to the phenetic clustering and additive distance analyses, in which only identical taxa were eliminated and the data sets consisted of 124 taxa, the parsimony analysis was performed on a reduced data set of 95 taxa. Both identical taxa and taxa that differed from others in the set only by possessing unique (and thus parsimony uninformative) alleles were eliminated. Because of the large numbers of character states (>32) in two of the characters, we used a computer with a 64 bit Alpha processor to perform a heuristic search, obtaining the starting tree using simple stepwise addition, searching for shorter trees using TBR branch swapping, with the maximum number of trees set to 150,000. The two species of *Typhlops* were designated as outgroups. The heuristic search yielded the maximum of 150,000 equally most parsimonious trees (length = 144), the strict consensus of which (not illustrated) was completely unresolved.

#### DISCUSSION

Our results indicate that the allozyme data published by Dowling et al. (1996)

are highly ambiguous concerning the higher-level phylogeny of snakes. This ambiguity exists regardless of whether the data are analyzed using phenetic clustering, additive distance methods, or character-based parsimony methods. Under all three approaches, thousands of trees explain the data equally well, or nearly so, and the consensus of those trees exhibits little to no resolution, particularly concerning the deeper nodes. Given the ambiguity of the data, it is worthwhile to consider, first, how Dowling et al. (1996) obtained a single, highly resolved tree, and, second, why that tree exhibits general agreement with traditional snake taxonomy.

A single, highly resolved tree presumably was obtained because the original analysis did not reveal the existence of alternative (equivalent) trees. Identical values in a distance matrix imply the existence of alternative clustering pathways and thus alternative trees. However, many software implementations of UPGMA and related methods, including the one in the TAXAN package used by Dowling et al. (1996), do not explicitly reveal the existence of alternative trees. Instead, ties are broken arbitrarily. During each cycle in the clustering sequence, if two or more taxa are equally (and minimally) distant from a third taxon, the algorithm arbitrarily clusters one of these equally distant pairs first, typically based on the input order of the taxa (Hart, 1983; see also Farris et al., 1996). The alternative clustering sequence, which may result in a different branching pattern (i.e., a different tree), is not explored. Therefore, the tree produced may be only one member of a set of equivalent trees. For the data analyzed in this paper, there are both thousands of ties and thousands of equivalent trees.

The reason that the single tree of Dowling et al. (1996) exhibits general agreement with traditional ideas about snake taxonomy is that the equivalent trees do not differ radically from one another, so that any arbitrarily selected member of the set would probably agree, more or less, with traditional taxonomy. To demonstrate this, we measured the dissimilarity between the published tree and the 1000



equivalent UPGMA trees found by randomly breaking ties using the symmetric difference metric of Penny and Hendy (1985), as implemented in PAUP\* version 4.0b2. Symmetric differences ranged from 77–141 (0.318–0.583). We then found the tree that differed most from the published tree and compared it with the published tree in terms of congruence with the traditional taxonomy of snakes, as described by Dowling et al. (1996) and Dowling and Duellman (1978).

Even this maximally different tree agreed reasonably closely with traditional snake taxonomy, and more importantly, it did not seem to agree any less well than the published tree. Dowling et al. (1996: their Table VI) recognized 30 groups based on their tree. In 17 cases, the groups were identical in composition to groups on the maximally different tree, which therefore matched traditional taxonomy equally well (or poorly); in three cases, the group on their tree agreed better with traditional taxonomy; in five cases, the group on the most different tree agreed better; and in five other cases, relative agreement was equivocal either because both trees matched traditional taxonomy poorly (but not identically) or because each tree matched better in some respects and worse in others. Because the tree that is most different from the published tree fits traditional snake taxonomy reasonably well, it seems reasonable to expect that the same will be true of most, if not all, of the equivalent trees. Nevertheless, even minor differences among individual trees can sum to a high level of ambiguity when there are many equivalent trees. This appears to be the situation with the snake allozyme data, for which there are thousands of equivalent trees and the consensus tree (Fig. 2) is poorly resolved.

The ambiguity of the snake allozyme data reanalyzed here is presumably related to the rate of evolution of the four loci surveyed, which appears to be too rapid for the question being addressed. Those loci have been characterized as slowly-evolving, and they may indeed be evolving slowly relative to other loci commonly surveyed using protein electrophoresis. Nev-

ertheless, they are evolving relatively rapidly in the context of the higher-level phylogeny of snakes. Rapid evolution is manifested in the large numbers of alleles (25 to 43) at each locus and in the fact that more than half of the entries in the distance matrix exhibit the maximum possible distance value of 1.00 (Table 1), indicating that the taxa share no alleles at any of the four loci. The loci in question may be useful for analyzing relationships within smaller clades of snakes, but they are highly ambiguous concerning the relationships within snakes as a whole, particularly those related to the deeper divergences in the history of that clade.

*Acknowledgments.*—We thank R. Highton and C. A. Hass for generously providing original data and results, F. J. Rohlf for assistance with NTSYSpc, including increasing the tied-tree limit of the program, D. L. Swofford for providing access to test versions of PAUP\*, and J. C. Wilgenbusch and J. A. McGuire for help with analyses performed on 64 bit computers.

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Accepted: 10 October 1999  
 Associate Editor: Stephen Tilley

*Herpetologica*, 56(3), 2000, 332–342  
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## MORPHOMETRIC VARIATION AMONG LARVAE OF FOUR SPECIES OF LUNGLESS SALAMANDERS (CAUDATA: PLETHODONTIDAE)

GAYLE LIVINGSTON BIRCHFIELD<sup>1</sup> AND RICHARD C. BRUCE<sup>2</sup>

*Highlands Biological Station, Highlands, NC 28741, USA and Department of Biology, Western Carolina University, Cullowhee, NC 28723, USA*

**ABSTRACT:** Several linear dimensions and body masses were recorded for larvae of four closely-related, similar species of lungless salamanders (family Plethodontidae) that occur over a broad gradient of elevation and habitat in the southeastern United States. The species were *Gyrinophilus porphyriticus*, *Pseudotriton ruber*, *P. montanus*, and *Stereochilus marginatus*. The goals were to evaluate the usefulness of morphometrics in species identification and to examine trends in variation in relation to habitat utilization in the four species. To remove the effect of variation in body size, log<sub>e</sub>-transformed values of the variables were plotted against log<sub>e</sub> snout-vent length, and the residuals generated by these plots were used as the dependent variables in all analyses. Discriminant function analyses were only partially successful in classifying larvae by species. Multivariate analyses of variance (MANOVA) failed to reveal any pronounced trends in morphology related to the stream- and pond-type categories of larval salamanders recognized by herpetologists. However, the slender habitus of larvae of the lowland species, *S. marginatus*, may represent adaptation to sphagnum mats in ponds and sluggish streams in the Coastal Plain. The slender body and reduced eyes of larvae of the montane *G. porphyriticus* are probably adaptations to a subsurface mode of life in springs and headwater streams. It is proposed that lunglessness, herein considered a larval adaptation to life in mountain streams in plethodontids, is one factor that constrains adaptive diversification in this clade, relegating larvae to a bottom-dwelling mode of life in lentic as well as lotic habitats.

*Key words:* Caudata; *Gyrinophilus porphyriticus*; Larvae; Morphology; *Pseudotriton montanus*; *Pseudotriton ruber*; Salamanders; *Stereochilus marginatus*

HEMIDACTYLINAE salamanders of the genera *Gyrinophilus*, *Pseudotriton*, and *Stereochilus* are thought to constitute a monophyletic lineage containing the least-derived species of the family Plethodontidae (Lombard and Wake, 1986; Wake, 1966). Putative plesiomorphic characters of this lineage include a biphasic life cycle

and a lengthy larval phase, ranging from 1.5–2.5 yr in *Stereochilus marginatus* (Bruce, 1971), *Pseudotriton montanus* (Bruce, 1974, 1978), and *P. ruber* (Bruce, 1972, 1974; Semlitsch, 1983) to as long as 4–5 yr in *Gyrinophilus porphyriticus* (Bruce, 1980). The cavernicolous *G. pal-leucus* and *G. subterraneus* are paedomorphic or nearly so (Besharse and Holsinger, 1977; Brandon, 1971). Thus, the larval period is an important phase of the life cycle of all these species.

In the southeastern United States, *G. porphyriticus*, *P. ruber*, *P. montanus*, and

<sup>1</sup> PRESENT ADDRESS: Division of Biological Sciences, University of Missouri, Columbia, MO 65211, USA.

<sup>2</sup> CORRESPONDING ADDRESS: Department of Biology, Western Carolina University, Cullowhee, NC 28723, USA.

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